



Co-relationship between *Escherichia coli* in broiler cellulitis and liver lesions

R. M. Silva^{a*} , I. M. M Silva^{a,b} , M. C. Jesus^b , M. D. B. Fernandes^a , F. S. Oliveira^a
and J. Evêncio-Neto^c

^aUniversidade Federal do Recôncavo da Bahia – UFRB, Complexo Multidisciplinar de Estudo e Pesquisa em Saúde, Santo Antônio de Jesus, BA, Brasil

^bCentro de Ciências Agrárias Ambientais e Biológicas – UFRB, Programa de Pós-graduação em Microbiologia Agrícola, Cruz das Almas, BA, Brasil

^cUniversidade Federal Rural de Pernambuco, Departamento de Histopatologia, Recife, PE, Brasil

*e-mail: ricardomendes@ufrb.edu.br

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Abstract

Pathogenic strains of *Escherichia coli* may invade the subcutaneous tissue of poultry and cause cellulitis, whilst the pathogen may also cause lesions in internal organs such as the liver. Current paper co-relates *Escherichia coli* and virulence genes characteristic of Avian Pathogenic *Escherichia coli* (APEC) in broilers' cellulitis and liver lesions. One hundred carcasses were retrieved from the production chain in an avian abattoir in the state of Bahia, Brazil, between August 2013 and January 2014, due to detection of cellulitis lesions. Cellulitis and liver samples were retrieved aseptically to quantify *E. coli* by Petrifilm™ count fast method (3M Company) (AOAC 998.8). Virulent genes *iss* and *iutA* were removed from *E. coli* isolates by Polymerase Chain Reaction (PCR). *Escherichia coli* was isolated from 82.0% of broilers removed from the production chain and the bacterium was concomitantly detected in cellulitis and liver lesions in 40.0% of broilers. *E. coli* counts ranged between 1.00 and 4.73 log CFU/g in liver lesions and between 2.00 and 9.00 log UFC/g in cellulitis lesions. Virulent genes *iutA* and *iss* were detected in 97.56% and 89.02% of *E. coli* isolates, respectively. Genotype analysis demonstrated the concomitant amplification of genes *iutA* and *iss* in 60.0% (n=40) of samples of cellulitis and liver lesions in which the simultaneous isolation of *E. coli* occurred. There was a positive and significant co-relationship ($r=0.22$; $p<0.05$) between the variables occurrence of *E. coli* isolated from liver samples and the occurrence of *E. coli* isolated from cellulitis lesions. There were also positive and significant co-relationships between populations of *E. coli* from liver isolates and cellulitis lesions ($r=0.46$; $p<0.05$) when *E. coli* isolated in the liver and in cellulitis lesions was detected. Since results showed a relationship between *E. coli* in cellulitis and liver lesions and possible systemic infection, the occurrence of cellulitis lesions as a criterion for total discarding of carcass may be suggested.

Keywords: sanitary inspection, enterobacteriaceae, microbial genetics, food and nutritional safety.

Correlação entre a presença de *Escherichia coli* em lesões de celulite e fígados de frango

Resumo

Cepas patogênicas de *Escherichia coli* podem invadir o tecido subcutâneo das aves e provocar celulite aviária e este patógeno pode provocar lesões nos órgãos internos, como o fígado. Desta forma, objetivou-se correlacionar a presença de *Escherichia coli* e os genes de virulência característicos de *Escherichia coli* Patogênica para Aves (APEC) nas lesões de celulite e nos fígados dos frangos. Entre agosto de 2013 a janeiro de 2014, foram retiradas 100 carcaças da linha de produção por apresentarem lesões de celulite em um matadouro avícola da Bahia (Brasil). Foram coletadas amostras de celulite e fígados de frango assepticamente para quantificação de *E. coli* pelo método rápido de contagem Petrifilm™ (3M Company) (AOAC 998.8). Em seguida foi realizada a pesquisa dos genes de virulência *iss* e *iutA* nos isolados de *E. coli* utilizando a Reação em Cadeia da Polimerase (PCR). *Escherichia coli* foi isolada em 82,00% das aves retiradas da linha de produção e a bactéria foi detectada concomitantemente nas lesões de celulite e fígado em 40,00% das aves. As contagens de *E. coli* variaram de 1,00 a 4,73 log UFC/g nos fígados e de 2,00 a 9,00 log UFC/g nas lesões de celulite. Os genes de virulência *iutA* e *iss* foram encontrados em 97,56% e 89,02% dos isolados de *E. coli*, respectivamente. A análise genotípica revelou a amplificação concomitante dos genes *iutA* e *iss* em 60,00% (n=40) das amostras de lesões de celulite e fígado nas quais houve o isolamento simultâneo de *E. coli*. Foi observada correlação positiva e significativa ($r=0,22$; $p<0,05$) entre as variáveis ocorrência de *E. coli* isolada das amostras dos fígados e

ocorrência *E. coli* isolada das lesões de celulite e, nos casos em que foi detectada a ocorrência de *E. coli* isolada em fígado e lesões de celulite, correlações positivas e significativas também foram evidenciadas entre as populações de *E. coli* dos isolados dos fígados e das lesões de celulite, ($r=0,46$; $p<0,05$). Assim ficou evidenciada a relação entre *E. coli* presente nas lesões de celulite e no fígado e uma possível infecção sistêmica, desta forma, sugere-se que a presença de lesões de celulite seja utilizada como critério para o descarte total da carcaça.

Palavras-chave: inspeção sanitária, enterobacteriaceae, genética microbiana, segurança alimentar e nutricional.

1. Introduction

The avian industry has a robust economic impact since it produces low-cost animal protein. In 2017, Brazil produced 13,056,000 tons of broiler meat, ranking second worldwide. Further, Brazil exported 4,320,000 tons of broiler meat and derived products, ranking first in export worldwide (ABPA, 2018). However, Brazil has to increase its production efficiency, meet meat quality criteria and other requirements related to animal well fare and the environment so that such ranking may be maintained (Paschoal et al., 2012).

In spite of good performance, poultry culture has serious avian health issues to due to the industrial scale in broiler breeding. In fact, cutaneous diseases, particularly avian cellulitis, have become more and more frequent and have produced liabilities to poultry production, caused by the partial or total discard of carcasses in abattoirs (Vieira et al., 2014).

The main characteristics of broiler cellulitis are subcutaneous inflammation, especially on thighs and abdomen, and microscopic changes, such as skin thickening and irregularities with color alterations and with the formation of yellow plates detected under the skin (Ghanbarpour et al., 2010). Poultry cellulitis is mainly caused by inadequate management and nutrition and through pathogens, such as *Escherichia coli*, which invade the organism from a continuous solution in the skin (Vieira et al. 2006). Research on virulence genes in *E. coli* isolates identifies pathogenic strains in samples of several types of food (Barbosa et al., 2019; Silva et al., 2012) and is an important strategy to diagnose pathogenic strains.

Studies by Gomis et al. (2000) have shown that cellulitis-struck broilers may have also lesions in the heart, aerial pathways, bones, joints and liver. Within the internal organs, the liver may provide relevant information on the occurrence of systemic illnesses even though the etiology of several hepatic lesions cannot be pinpointed (Abdul-Aziz et al., 2016; Swayne, 2013).

Underscoring public health, the Ministry of Agriculture, Livestock and Supply insists that any organ or any segment in broiler carcass affected by an inflammation process should be discarded and, if there is evidence of systemic issues, the carcass and the viscera should be totally rejected (Brasil, 1998).

Due to the lack of studies that co-relate microbiological and molecular findings in broilers' cellulitis and liver lesions, current analysis co-relates *E. coli* and virulence

genes characteristics of Avian Pathogenic *Escherichia coli* (APEC) in broilers' cellulitis and liver lesions.

2. Material and Methods

One hundred samples of cellulitis and liver lesions of broilers (*Gallus gallus*), with cellulitis lesions were collected in a poultry abattoir in the Reconcavo Sul of the state of Bahia (Brazil), monitored by the Agriculture and Livestock Agency of Bahia (ADAB), between August 2013 and January 2014.

Samples were collected aseptically with a scalpel lamina and placed in sterile containers. They were identified and transported in an isothermal icebox and immediately sent to the Center for Health Studies and Research at the Center of Health Sciences of the Universidade Federal do Recôncavo da Bahia.

Escherichia coli was isolated by fast count method in Petrifilm™ plates (3M Company), with Petrifilm EC™ Plate (AOAC 998.8), following instructions by manufacturer. Colony counts were undertaken by colony counter CP600 Plus (Phoenix®) which calculated the number of log CFU/g (Silva et al., 2017).

At first, up to three colonies typical of *E. coli* were isolated, originating from Petrifilm EC™ plates (3M Company). Each colony was inoculated with a platinum handle or needle in a microtube with Brain Heart Infusion (BHI) broth, at $35\pm 1^\circ\text{C}$ for $24\pm 2\text{h}$. After this period, 2mL glycerol 15% were added and samples were frozen at minus 20°C for DNA extraction later on.

For DNA extraction, samples were inoculated in BHI broth and incubated at $35\pm 1^\circ\text{C}$ for $24\pm 2\text{h}$. They were then centrifuged for 5 min, at 13,500 rpm. Supernatant was discarded and 800µL of ionized water were added. Homogenization and centrifuge were undertaken in the same conditions as described above. Supernatant was once again discarded, 80µL of deionized water were added and the resulting content homogenized. Samples were then heated at 96°C for 10 min; 2µL of supernatant was placed in a microtube with 18µL of ultrapure water (Hexapur™). DNA samples were stored at minus 20°C until analysis.

DNA was quantified by spectrophotometry (BioPhotometer D30 Eppendorf™) and standardized with final concentration of 50 ng/10µL. Virulence genes prevalent in pathotypes APEC (*iss* and *iutA*) were analyzed by Polymerase Chain Reaction (PCR).

Table 1 described the characteristics of primers.

The mix was prepared in an aseptic chamber and 24µL were distributed in 0.2mL polypropylene tubes, while

1µL of each DNA sample was added per tube. Ultrapure water (Hexapur™) was employed for negative control; standard strains APEC (ATCC 25922), provided by the Oswaldo Cruz Foundation of Rio de Janeiro, were used as positive control. PCR was undertaken in Mastercycler (Amplitherm™) thermocycler.

Further, 10µL of the amplified product, positive and negative controls, and 2µL of molecular weight of 100pb DNA ladder were added to each well of agar gel 2%, stained with ethidium bromide (10 mg/mL). Separation was done by electrophoresis with GSR@1000STD at the following conditions: 100 minutes, 60V, 37mA and 2W. Results were reported on ultraviolet trans-illuminator (Loccus®).

Statistical analysis was performed with SPSS 22.0, featuring means and standard deviation in the descriptive analysis of data and percentage frequencies for qualitative variables. Pearson's Correlation Test was employed to compare frequencies of occurrence in the isolation of *Escherichia coli* of cellulitis and liver lesions and of the quantification of *Escherichia coli* isolates in cellulitis and liver lesions at 0.05 significance level.

3. Results and Discussion

Escherichia coli was isolated in 82.0% of cellulitis samples and in 45.0% of liver samples. Bacterium was detected concomitantly in cellulitis and liver lesions in 40.0% of broilers (Table 2).

Escherichia coli counts in liver isolates ranged between 1.00 and 4.73 log CFU/g and in cellulitis lesions between 2.00 and 9.00 log CFU/g, corroborating results by Vieira et al. (2013) who isolated *Escherichia coli* strains in 50 broiler samples from an abattoir in the state of Rio de Janeiro, where the bacterium occurred simultaneously in 50.0% (n=25) of cellulitis and liver lesions.

Contrasting results were provided by Gomis et al. (2001) in a Canadian research. The authors isolated *Escherichia coli* in 237 cellulitis samples. The bacterium was also isolated in only three samples of the above lesions in the liver with macroscopic lesions. However, the lack of macroscopic finds failed to guarantee the organ's harmlessness. In fact, Silva et al. (2012) isolated *Escherichia coli* in 60% (n=30) of samples of broilers' livers without any macroscopic alterations, in the Recôncavo da Bahia region.

There was a positive and significant co-relationship ($r=0.22$; $p<0.05$) between variable occurrence of *Escherichia coli* isolated from liver samples and *Escherichia coli* isolated from cellulitis lesions, by Pearson's linear correlation coefficient.

Brito et al. (2003) stated that *Escherichia coli* strains, isolated from cellulitis, were more associated with the emergence of sepsis than isolates of other clinical manifestations of colibacillosis. Johnson et al. (2001) experimentally inoculated *Escherichia coli* in broilers to favor the emergence of cellulitis lesions and detected other clinical manifestations of colibacillosis in most contaminated broilers, particularly perihepatitis, pericarditis and aerosaculitis.

Another positive and significant co-relationship for samples in which *E. coli* was isolated in the liver and in cellulitis lesions occurred between *E. coli* counts of livers and cellulitis lesions ($r=0.46$; $p<0.05$). Consequently, the greater the occurrence of cellulitis lesions, the greater is the bacterial population in the livers, corroborating results by Vieira et al. (2013). The authors suggest that *Escherichia coli* strains penetrate the animal organism through the cellulitis lesion and reach the internal organs.

Virulence genes *iutA* and *iss* occurred in 97.56% and 89.02% of *E. coli* isolates, respectively. Genotype analysis revealed the concomitant amplification of genes *iutA* and *iss*, characteristic of APEC, in 60.0% (n=40) of samples of cellulitis and liver lesions in which the simultaneous

Table 1. Characteristics of PCR primers in samples of cellulitis and liver lesions of broilers from the avian abattoir of the Recôncavo da Bahia (Brazil). 2014

Gene	Sequence of oligonucleotides	Amplicom (MW)	Function
<i>iss</i> *	GTGGCGAAAAGTAGTAAACAGC CGCCTCGGGGTGGATAA	760	Resistance to serum
<i>iutA</i> **	GGCTGGACATCATGGGAACTGG CGTCGGGAACGGGTAGAATCG	302	Iron acquisition

*Source: Knöbl et al. (2012); **Source: Johnson et al. (2008). MW- Molecular Weight.

Table 2. Frequency of *Escherichia coli* and virulence genes in samples of cellulitis and liver lesions of broilers from avian abattoir of the Recôncavo da Bahia (Brazil). 2014

Find /Site	Cellulites	Livers	Cellulites and Livers
<i>Escherichia coli</i>	82/100*	45/100*	40/100*
<i>iutA</i>	80/82	43/45	38/40
<i>Iss</i>	73/82	29/45	24/40
<i>iutA</i> and <i>iss</i>	24/40	24/40	24/40

*100 number of carcasses with cellulitis lesions retrieved from broilers in the production chain of avian abattoir in the Recôncavo da Bahia.

isolation of *E. coli* occurred. Azam et al. (2019) researched broilers with colibacillosis in Pakistan and detected that the genes were the most prevalent in APEC, with 74.6% and 84.0%, respectively.

Brito et al. (2003) identified *Escherichia coli* strains originating from cellulitis lesions, with similar virulence factors in 52 batches of broilers from the southern states of Brazil. The authors suggested the pathogen's endemic dissemination.

According to Rodriguez-Siek et al. (2005), chicken meat is a possible vector for APEC dissemination, or rather, an extra-intestine *E. coli* (ExPEC) with zoonotic potential. According to Maluta et al. (2014), APEC shares homology of genomic sequences between strains of other human ExPECs (NMEC and UPEC), which are important causes of infections in humans.

In the wake of imminent risk by APECs to human health and in collaboration with Vieira et al. (2014) who stated that the partial removal of cellulitis lesions merely lessens the carcass's repugnant aspect, more aesthetic than hygienic, failing to contribute towards the elimination of contamination, one perceives that the official criteria for discarding carcasses should be revised. Current authors suggest that cellulitis lesions should be the criterion for the carcass's total and not merely partial discard. This is due to the possibility of food unsafeness caused by the intake of chicken meat and derived products.

Positive and significant co-relationship between *E. coli* isolation in cellulitis and liver lesions from broilers bred and slaughtered in the Reconcavo da Bahia region suggests that broilers' cellulitis should be the initiating factor of the pathogenic infectious process which may attack other organs, especially the liver.

Concomitant amplification of the virulence factors *intA* and *iss* in most cases in which *Escherichia coli* was simultaneously isolated in cellulitis and liver reinforces the possibility that pathotype APEC would occur in the two tissues. This fact strengthens the hypothesis that the bacterium infects the animal through skin lesions and then attacks the liver. The above boils down to a systemic and not a local factor with regard to results on cellulitis lesions.

The relationship between *E. coli* in cellulitis and liver lesions and a possible systemic infection is evidenced. It may be suggested that the occurrence of cellulitis lesions may be employed as a criterion for the total discarding of the carcass. The health of the consumer and the elimination of risks within the broilers' production chain are ensured.

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